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Identification of evodiamine as the bioactive compound in evodia (*Evodia rutaecarpa* Benth.) fruit extract that activates human peroxisome proliferator-activated receptor gamma (PPARγ)

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ABSTRACT

The dried unripe fruit from *Evodia rutaecarpa* Benth., known as Wu zhu yu in China, has long been used in traditional Chinese medicine. In this research, we provide evidence that evodia fruit extract activates peroxisome proliferator-activated receptor gamma (PPARγ) and, as identified through HPLC fractionation and mass spectroscopy, the activating phytochemical is evodiamine. Evodiamine was shown to bind to and activate PPARγ. It was also shown to activate PPARγ-regulated gene expression in human hepatoma cells similar to known PPARγ ligands and that the expression was blocked by a PPARγ specific antagonist.

1. Introduction

The dried unripe fruit from *Evodia rutaecarpa* Benth., known as Wu zhu yu in China, has long been used in traditional Chinese medicine for the treatment of gastrointestinal disorders (abdominal pain, dysentery), headache, amenorrhea, and postpartum hemorrhage [1–3]. It also has a history of use as a cardiotonic, a central stimulant with transient hypertensive and positive inotropic and chronotropic effects [4,5]. In phytochemical studies, the indoloquinazoline alkaloids from the Evodia fruit (*Evodiae fructus*), evodiamine and rutaecarpine, have been linked to these cardiotonic effects via activation of the vanilloid receptor [6] and subsequent production of calcitonin gene-related peptide [7]. These compounds also act on other cardiovascular-related targets, rutaecarpine inhibits platelet aggregation and evodiamine is a vasorelaxant [8,9].

Rutaecarpine and evodiamine have also been shown to act at additional biological targets. In inflammation, rutaecarpine directly inhibits COX-2 enzymatic activity [10], while evodiamine inhibits COX-2 gene expression via an NF-κB/Akt dependent pathway [11,12]. In aberrant cell growth, evodiamine has been shown to inhibit cellular growth, invasion, and metastasis of a wide variety of tumor cells, while having little toxicity on normal human peripheral blood cells [13]. In energy metabolism, evodiamine affects biological targets involved in obesity and adipocyte maturation while reducing diabetic complications in vivo [14,15]. It has been shown to block adipogenesis by activating ERK thru EGFR activation and PKCα [15,16].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand activated nuclear receptor that directly binds to and regulates gene expression (for review see [17]). Its activation

Abbreviations: COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; FABP1, human fatty acid binding protein 1; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; HPRT1, hypoxanthine phosphoribosyltransferase; LBD, ligand-binding domain; PCK1, phosphoenolpyruvate carboxykinase 1; PPAR, peroxisome proliferator-activated receptor; PKCα, protein kinase C alpha; TR-FRET, time-resolved fluorescence resonance energy transfer; UAS, upstream activating sequence.

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affects energy metabolism as well as inflammation and tumor growth. In adipocytes, PPARγ activation stimulates lipids and glucose uptake, leading to lipid storage. However, evodiamine blocks PPARγ induced adipogenesis [18]. Therefore, evodiamine is not an obvious PPARγ agonist. PPARγ ligands have a positive effect on insulin action and improve glucose tolerance in diabetic animals and humans, as well as improvements in inflammation and cognitive function [17]. These observations are consistent with the effects seen for evodiamine in obesity, diabetic, and Alzheimer's disease animal models [14,15,19].

In this research, we showed that evodia fruit extracts demonstrate a robust response in a PPARγ luciferase reporter assay. We further identified through HPLC fractionation and mass spectroscopy that the activating phytochemical is evodiamine.

2. Materials and methods

2.1. Chemicals and reagents

Evodiamine, rutaecarpine, and PPARγ agonists, rosiglitazone, and troglitazone were obtained from Sigma Corporation (St. Louis, MO). Evodiamine is a synthetic product (Sigma Corp.) and as such is a mixture of chiral forms [20,21]. The PPARγ antagonist, T0070907, and PPARα agonist, GW-7647, were from Tocris Biologicals (Minneapolis, MN).

Vectors pGL4.35 9XUAS luc2P and pFN26A BIND, and Fugene 6 were purchased from Promega Inc. (Madison, WI). Restriction enzymes were from New England Biolabs (Ipswich, MA), D-luciferin was from Biotium (Hayward, CA), and LanthaScreen TR-FRET PPARγ competitive binding assay was from Life Technologies (Grand Island, NY).

Dulbecco's minimum essential media (DMEM), Ham's F-12K (Kaighn's, F12K) media, Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS), pennicillin/streptomycin, and amphotericin B were purchased from Fisher Scientific (Pittsburgh, PA); hygromycin from EMD Millipore (Temecula, CA); and bovine serum albumin (BSA), G-418, and β-mercaptoethanol (BME) were purchased from Sigma Corporation (St. Louis, MO).

RNeasy Plus Mini kit, and quantitative polymerase chain reaction (qPCR) primer sets for human fatty acid binding protein 1 (FABP1), phosphoenolpyruvate carboxykinase 1 (PCK1), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), and the housekeeping gene, hypoxanthine phosphoribosyltransferase 1 (HPRT1) were purchased from Qiagen (Toronto, ON). First strand cDNA synthesis kit, iScript, and the real-time qPCR kit, SsoFast EvaGreen, were from Bio-Rad Laboratories (Hercules, CA).

2.2. Evodia rutaecarpa fruit extracts

E. rutaecarpa fruit was harvested from plants cultivated in China. The fruit was dried, milled into a homogenous feedstock, and extracted at room temperature for 24 h in methanol or chloroform using a ratio of 1:10 (fruit:solvent). The extract was filtered, and solvent was removed by drying under nitrogen. The resulting solids were quantified by mass, suspended in the original extraction solvent, aliquoted, re-dried, sealed, and stored at −80 °C. Before biological testing, the dried solids were suspended in 70:30 DMSO:water, shaken for 2 h, and diluted in cell culture media prior to assay.

Powdered *E. rutaecarpa* fruit extract was suspended in methanol:water (50:50) at 50 mg/mL, sonicated for 10 min, then filtered through a 0.45 μm PVDF membrane syringe filter into an autosampler vial for UPLC fractionation and LC-MS analysis.

2.3. UPLC fractionation and LC-MS analysis

Chromatographic separation was performed on an Acquity UPLC-H chromatograph with photodiode array detector (PDA) and XBridge Shield RP18 (5 μm, 4.6 × 250 mm) column. The mobile phase solutions used for gradient separation were: A, 0.1% acetic acid in water and B, 0.1% acetic acid in acetonitrile. The mobile phase gradient (A:B), at ambient temperature and a flow rate of 0.8 mL/min, was initially set at 95:5 and linearly changed to 0:100 from 0 to 30 min, held for 2 min, and then, returned to initial conditions at 32.1 min and held till 35 min. The injection volume was 40 μL for both samples and standards and the chromatogram recorded between 210 and 800 nm at a 1.2 nm resolution.

The effluent was split after the PDA, with the majority going to a fraction collector configured for 96 well plates with 2 mL/well volume. Fractions were collected at 20 s intervals for 32 min. The plates were frozen at −80 °C overnight, and the solvent was removed by freeze drying. The sample plates were sealed and stored dry at −20 °C until assayed for activity.

The remaining effluent was directed to a Waters Synapt G2 mass spectrometer equipped with an electrospray ion source and operated at 25 V cone voltage. Accurate mass spectra were collected in positive ion mode from *m/z* 100 to 1200 at 0.5 s/scan, with alternating spectra collected at 0 V and 20 V collision energy in the transfer cell (MS^e mode). The mass spectra, UV spectra, and fractions were time aligned for identification of active compounds.

2.4. Plasmid constructs

To study the activation of PPARγ and PPARα in a luciferase reporter assay, Gal4-PPARγ-ligand-binding domain (LBD) and Gal4-PPARα-LBD vectors were created in a modified pFN26A (BIND) vector (Promega, Inc.). The barnase gene was excised and a short cloning region (5′-cgcagagctcaaaagcg-3′) was inserted at the PvuI/EcoRI site to create pBIND3. The PPARγ-LBD (residues 203–477) was qPCR amplified from human MGC: 5041 (pSPORT6; Open Biosystems, Inc.) using forward and reverse primers 5′-acgatcgaacagctgaatccagagtccgctga-3′ and 5′-tctagactagtacaag tccttgtagatctcctgcaggagc-3′, incorporating pvuI and xbaI sites at the 5′ and 3′ ends, respectively. The PPARγ-LBD fragment was then ligated into the pBIND3 PvuI/XbaI site forming a Gal4-PPARγ-LBD fusion protein. Similarly, the PPARα-LBD (residues 201–468) vector was created using forward and reverse primers, 5′-cgatcgcagacctcaaatctctgggc-3′ and 5′-gaattcagtacatgtctctgtagatctcttgc-3′, to amplify the PPARα-LBD from mouse MGC:18607 (Invitrogen). The PPARα-LBD fragment was then ligated into the pBIND3 PvuI/EcoRI site forming a Gal4-PPARα-LBD fusion protein.

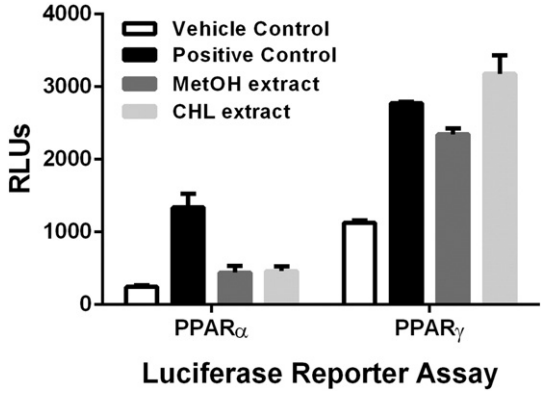

Fig. 1. Effect of chloroform (CHL) and methanol (MetOH) evodia fruit extracts (50 μg/ml) on PPARγ-LBD and PPARα-LBD luciferase assays. Positive controls for the PPARα and PPARγ assays were GW-7647 (2 μM) and troglitazone (10 μM), respectively. Luciferase activity was determined at 18 h. The data shown is a representation of 3 separate experiments conducted under the same conditions. Each column represents the mean ± SD, with $n = 2$.

2.5. Cell culture and stable cell lines

Human hepatoma (HepG2) and Chinese hamster ovary (CHO-K1) cells were purchased from ATCC (Manasas, VA) and grown in a 37 °C, 5% CO_2 incubator in DMEM and F12K media, respectively. All growth media was supplemented with 10% FBS, penicillin/streptomycin (100 U/100 μg/mL), and amphotericin B (250 μg/mL).

Dual vector stable cell lines for PPARγ and PPARα assays were generated by first transfecting CHO-K1 cells with pGL4.35 [luc2P/9XGAL4 UAS/Hygro] vector using Fugene 6 according to manufacturer's instructions. Hygromycin (300 μg/mL) supplemented media was used to screen for monoclonal cell lines 24 h after transfection, and selected monoclonal cell lines were screened by dilution method until there was no further cell death. The resulting stable cell line was maintained using growth media supplemented with hygromycin (150 μg/mL).

PPARγ and PPARα cell lines were created by transfecting the stable pGL4.35 [luc2P/9XGAL4 UAS/Hygro] vector CHO-K1 cell line with pBIND3 Gal4-PPARγ-LBD or pBIND3 Gal4-PPARα-LBD vector and selecting for pBIND3 Gal4-PPAR-LBD containing cells with G418 (600 μg/mL) supplemented media. The resulting stable cell lines were maintained using growth media supplemented with both hygromycin (150 μg/mL) and G418 (300 μg/mL).

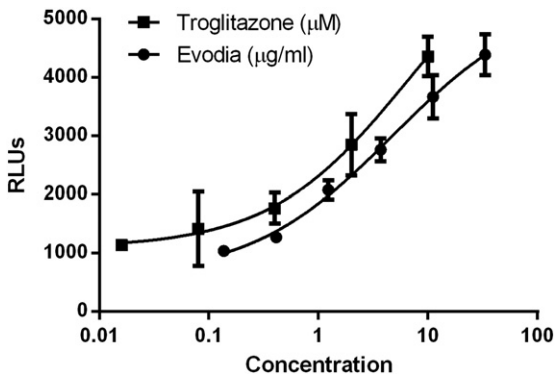

Fig. 2. Effect of increasing concentrations of evodia fruit extract and the PPARγ agonist, troglitazone, on the PPARγ luciferase reporter assay. Luciferase activity was determined at 18 h. Data points are mean ± SD, $n = 3$. The graph is representative of 3 separate experiments with similar results.

2.6. Luciferase reporter assay

Stably transfected CHO-K1 cells expressing the luciferase reporter were plated (2×10^4 cells/well) in white-walled, clear-bottom, 96-well plates and incubated overnight. The cells were then serum starved for 24 h and treated with select compounds at specified concentrations for 18 h in a 37 °C, 5% CO_2 incubator. Luciferase activity was quantified using a luciferase assay kit (Biotium, Inc., Hayward, CA). Briefly, cells were rinsed with 50 μL of DPBS, and then lysed with 20 μL lysis buffer for 20 min at room temperature. D-luciferin was added (100 μL/well) and light emission was read immediately on a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were plotted as relative light units (RLU) against test compounds using GraphPad Prism software (San Diego, CA).

2.7. LanthaScreen TR-FRET PPARγ competitive binding assay

To determine evodiamine binding to PPARγ-LBD, the LanthaScreen TR-FRET (time-resolved fluorescence resonance energy transfer) PPARγ competitive binding assay was performed according to the manufacturer's instructions. Briefly, increasing concentrations of select compounds were added to an assay mixture of GST-PPARγ-LBD fusion protein (lacking the DNA binding domain of PPARγ), fluoromone (a proprietary fluorescent pan-PPARγ ligand), and a terbidium-labeled GST antibody. Changes in fluoromone binding to the terbidium-labeled LBD complex were measured using a SpectraMax M5 spectrophotometer with excitation wavelength set at 340 nm and emission wavelength at 520 nm for fluorescein and 490 nm for terbium. The TR-FRET ratio was calculated by dividing the emission signal at 520 nm by the emission signal at 490 nm. Competition curves were plotted as TR-FRET ratio against concentration of test compounds using GraphPad Prism software.

2.8. Quantitative polymerase chain reaction

HepG2 cells were seeded at 1×10^6 cells/well in 6-well culture plates and grown for 24 h then, the growth media was replaced with media supplemented with 0.5% BSA in place of FBS and the cells incubated for an additional 24 h. The cells were then incubated for 18 h with specific PPARγ agonists with and without the addition of the PPARγ antagonist, T0070907. The antagonist was added 20 min prior to the agonists. The cells were rinsed with DPBS and total RNA purified using the RNeasy mini plus kit per manufacturer's instructions. Total RNA was quantified using the A_{260}/A_{280} ratio, diluted to 1 μg per reaction, and reverse transcribed using the iScript cDNA synthesis kit. Real-time qPCR reactions were completed using SsoFast EvaGreen qPCR mix on a CFX96 Real-Time Thermocycler (Bio-Rad). The reaction conditions were as follows: 95 °C for 30 s; 40 cycles of 58 °C for 5 sec; and 95 °C for 5 s. Fluorescent

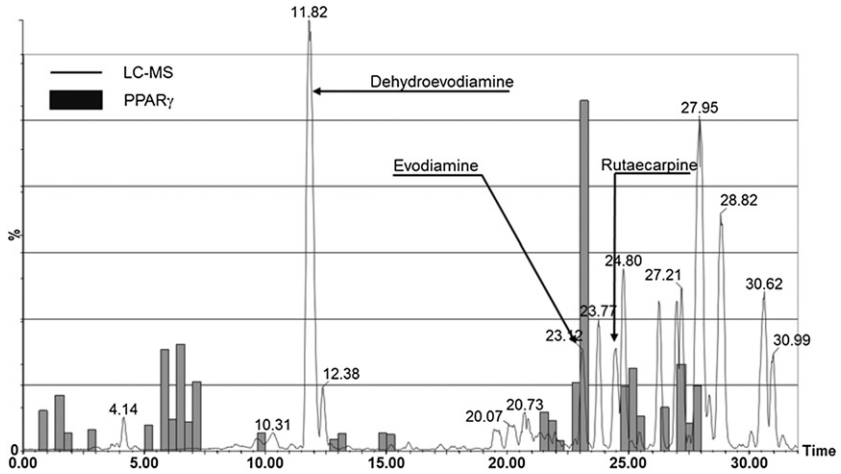

Fig. 3. PPARγ luciferase activity (■) and LC-MS chromatogram (—) of evodia fruit extract. The extract (50 mg/mL) was separated by reverse phase HPLC, and collected fractions tested for activity in the PPARγ luciferase reporter assay. Accurate mass spectral analyses were performed to identify the predominant phytochemicals in individual fractions.

detection was measured following completion of each cycle. Cycle times of PCK1, FABP1, and HMGCS2 were normalized to the housekeeping gene, HPRT1, prior to comparisons with control samples.

3. Results and discussion

One difficulty associated with the analysis of phytochemicals is their pleotropic nature [22,23]; they bind to and activate multiple biological targets thus affecting multiple signaling pathways. In our phytochemical testing strategy, we employed a luciferase reporter assay to isolate specific biological targets from other interfering signals. In this research, we ligated the specific ligand binding domains of PPARα and PPARγ to the DNA binding domain of Gal4 allowing us to use the Gal4/upstream activating sequence (UAS) signaling system [24]. The Gal4-PPAR-LBD constructs were then co-transfected with a UAS-luciferase construct into CHO-K1 cells. The results of the luciferase reporter assays were limited to the activation of the isolated LBD of PPARγ or PPARα. To further verify that the luciferase signal was specific to the activation of the PPAR-LBD, the evodia fruit extracts were also tested on a cell line stably transfected with only the UAS-luciferase construct.

Evodia fruit extracts demonstrated a robust response in the PPARγ-LBD luciferase assay that was not seen in either the PPARα-LBD luciferase assay (Fig. 1) or the UAS only control cell line (data not shown). Furthermore, the PPARγ luciferase response was greater for the evodia fruit extract prepared using the chloroform extraction method compared to the methanol

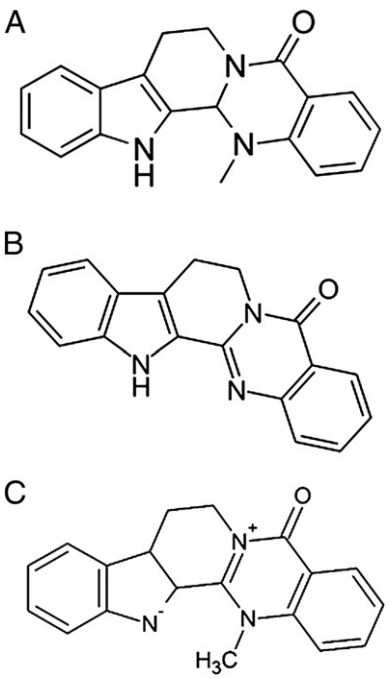

Fig. 4. The chemical structure of the primary phytochemicals in evodia fruit extract: evodiamine (A), rutaecarpine (B), and dehydroevodiamine (C).

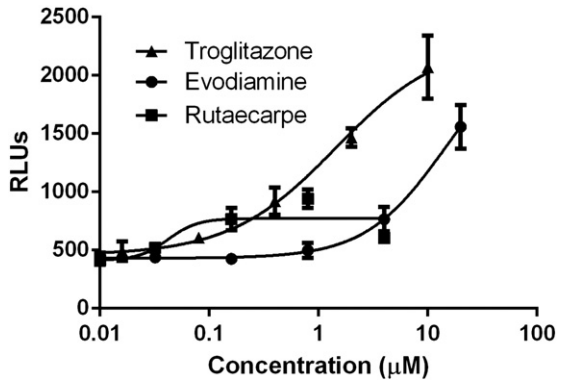

Fig. 5. Effect of increasing concentrations of evodiamine, rutaecarpine, and troglitazone in the PPARγ luciferase reporter assay. Luciferase activity was determined at 18 h. Data points are mean ± SD, $n = 3$. The graph is representative of 3 separate experiments with similar results.

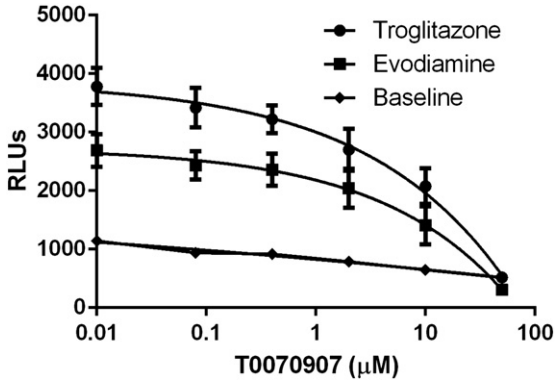

Fig. 6. Effect of increasing concentrations of the PPARγ antagonist, T0070907, on evodiamine (20 μM) and troglitazone (10 μM) activity in the PPARγ luciferase reporter assay. Luciferase activity was determined at 18 h. Data points are mean ± SD, $n = 3$. The graph is representative of 3 separate experiments with similar results.

extraction. The chloroform evodia fruit extract had a PPARγ luciferase response that was concentration-dependent and comparable in slope to that of the PPARγ agonist, troglitazone (Fig. 2). The EC_{50} values were approximately 5 μg/mL for evodia fruit extract and 5 μM (2 μg/mL) for troglitazone.

To determine which phytochemicals from the evodia fruit extract might activate PPARγ, an extract of evodia fruit was separated by reverse phase HPLC, and the fractions were tested for activity in the PPARγ reporter assay. Accurate mass spectral analysis of the effluent was conducted to identify the predominant phytochemicals in the individual fractions. Fig. 3 is an overlay of the LC-MS chromatographic data with the PPARγ luciferase activity in the collected fractions.

The chromatographic peak at retention time of 23.12 min., corresponding to fraction 70, was shown to have the greatest PPARγ luciferase activity. This fraction was analyzed by mass spectrometry and yielded a spectrum containing a single predominant ion at *m/z* 304.1443. This is consistent with an elemental composition of $C_{19}H_{18}N_3O$ (calculated exact mass 304.1450, −2.3 ppm difference). The isotopic distribution around the protonated molecular ion also supports the assigned formula. These data suggest that the observed ion is protonated evodiamine. A reference standard of evodiamine was analyzed for comparison and results confirmed that the retention times, mass spectra, and UV–Vis spectra of evodiamine and the unknown compound are identical; therefore, the compound was determined to be evodiamine.

Two structurally related compounds of evodiamine, dehydroevodiamine at 11.82 min and rutaecarpine at 24.49 min, showed no measureable PPARγ luciferase activity. The chemical structures of evodiamine, dehydroevodiamine, and rutaecarpine are presented in Fig. 4. These phytochemicals have been identified as the main alkaloids in *E. fructus* [25], and both evodiamine and rutaecarpine have been reported to have biological activity (for review see [26]).

Using purified commercial preparations of evodiamine and rutaecarpine, we confirmed that evodiamine yields a concentration-dependent response in the PPARγ luciferase reporter assay (Fig. 5). The rutaecarpine luciferase response was much less than evodiamine and was not concentration dependent. Furthermore, the rutaecarpine response in the UAS-luciferase only cell line was similar to that in the PPARγ reporter assay suggesting that the response was not specific to PPARγ. Evodiamine had no response in this UAS-luciferase only cell line (data not shown).

The specificity of the evodiamine response in the PPARγ luciferase reporter assay was confirmed by blocking the response with the PPARγ-LBD antagonist, T0070907 [27]. The evodiamine (20 μM) response was inhibited by T0070907 in a concentration-dependent manner (Fig. 6) similar to the T0070907 inactivation of the agonist, troglitazone (10 μM).

To determine if the evodiamine PPARγ luciferase response is through direct activation of the PPARγ-LBD we looked at PPARγ-LBD binding using the LanthaScreen TR-FRET PPARγ competitive binding assay. The PPARγ agonists, rosiglitazone and troglitazone, and antagonist, T0070907, were shown to decrease fluoromone binding to PPARγ-LBD (Fig. 7). Evodiamine binding was shown to be concentration-dependent. Rutaecarpine, which had a minimal response in the PPARγ-LBD luciferase reporter assay, did not inhibit the fluoromone binding to PPARγ-LBD (Fig. 7). The EC_{50} of troglitazone, rosiglitazone, and T0070907 were 100 nM, 40 nM, and 1 μM,

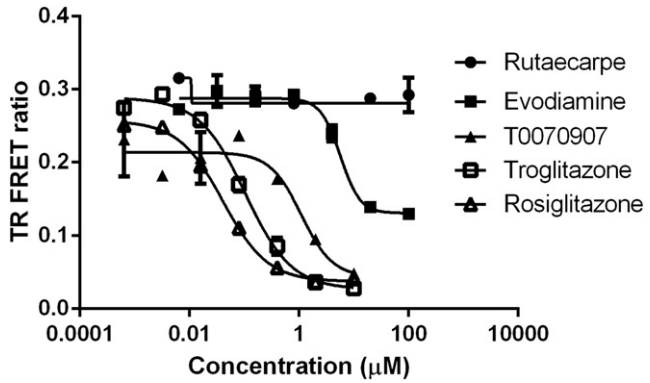

Fig. 7. Effect of evodiamine, rutaecarpine, troglitazone, rosiglitanzone, and T0070907 in a competitive ligand binding assay for PPARγ-LBD. Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) was performed. Troglitazone and rosiglitazone were PPARγ agonists and T0070907, a PPARγ antagonist. Data points are mean ± SD, $n = 2$. The graph is representative of 2 separate experiments with similar results. Linear curve fits were completed with GraphPad Prism using a 4 parameter non-linear curve fit.

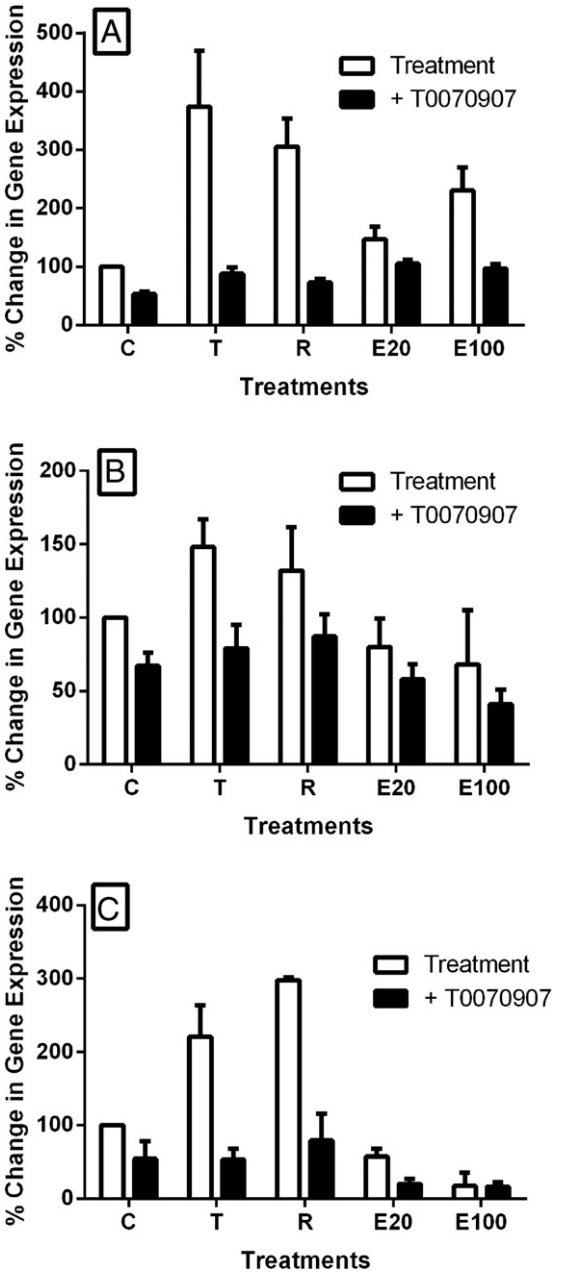

Fig. 8. The effect of evodiamine (E, 20 and 100 μM), troglitazone (T, 10 μM) and rosiglitazone (R, 10 μM) in the presence or absence of the PPARγ antagonist, T0070907 (10 μM), on mRNA expression levels of PCK1 (a), FABP1 (b), and HMGCS2 (c) in HepG2 cells as determined by qPCR. Vehicle (DMSO) treated HepG2 cells were used as controls (C). Each column represents the mean ± SD, $n = 3$.

respectively. The EC_{50} of evodiamine was 5 μM. Evodiamine acts like a partial agonist and does not completely displace fluoromone binding; however, we were unable to measure activity at concentrations higher than 100 μM because evodiamine interferes with the binding assay's fluorescent signal.

Having shown that evodiamine binds to and activates the PPARγ ligand binding domain, we next evaluated the effect of evodiamine on PPARγ-dependent gene expression. HepG2 cells were treated with evodiamine, total RNA extracted, and specific gene expression analyzed by qPCR. PPARγ agonists have been shown to increase gene expression of FABP1, PCK1, and HMGCS2 in HepG2 cells [28]. In these results, we show that evodiamine up-regulated PCK1 which was inhibited by T0070907 (Fig. 8a). Evodiamine was shown to slightly down-regulate FABP1 gene expression (Fig. 8b), and to completely down-regulate HMGCS2 expression (Fig. 8c). Both FABP1 and HMGCS2 were further down-regulated with the addition of T0070907. The PPARγ-LBD agonists, troglitazone and rosiglitazone, were both shown to up-regulate the expression of all three genes and this up-regulation was blocked in the presence of T0070907 (Fig. 8). The ability of evodiamine to bind to and modulate a variety of cellular targets might explain its varied effects on gene expression in HepG2 cells. Additional research is required to further elucidate the effect of evodiamine on PPARγ-responsive genes.

4. Conclusions

In summary, we have established that evodia fruit extract activates PPARγ as shown by specific activation of a PPARγ-LBD luciferase reporter assay and that the active phytochemical is evodiamine. The PPARγ agonist activity of evodiamine was confirmed in the luciferase assay and through a PPARγ-LBD binding assay. The evodiamine response was concentration-dependent and blocked by a specific PPARγ inhibitor, T0070907. Additional research will be necessary to further identify the effects of evodia fruit extract and evodiamine on PPARγ-mediated gene expression and biological responses.

Acknowledgments

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References

[1] Lee SH, Son JK, Jeong BS, Jeong TC, Chang HW, Lee ES, et al. Progress in the studies on rutaecarpine. Molecules 2008;13:272–300.
[2] Yu X, Wu DZ, Yuan JY, Zhang RR, Hu ZB. Gastroprotective effect of fructus evodiae water extract on ethanol-induced gastric lesions in rats. Am J Chin Med 2006;34:1027–35.
[3] Wang L, Hu CP, Deng PY, Shen SS, Zhu HQ, Ding JS, et al. The protective effects of rutaecarpine on gastric mucosa injury in rats. Planta Med 2005; 71:416–9.
[4] Li SC. National Research Institute of Chinese Medicine, Taipei, Pen-Tsao Kang Mu; 1976 1064.
[5] Liao JF, Chen CF, Chow SY. Pharmacological studies of Chinese herbs. (9) Pharmacological effects of *Evodiae fructus* (author's transl), Taiwan yi xue hui za zhi. J Formos Med Assoc 1981;80:30–8.
[6] Kobayashi Y, Hoshikuma K, Nakano Y, Yokoo Y, Kamiya T. The positive inotropic and chronotropic effects of evodiamine and rutaecarpine, indoloquinazoline alkaloids isolated from the fruits of *Evodia rutaecarpa*, on the guinea-pig isolated right atria: possible involvement of vanilloid receptors. Planta Med 2001;67:244–8.
[7] Jia S, Hu C. Pharmacological effects of rutaecarpine as a cardiovascular protective agent. Molecules 2010;15:1873–81.
[8] Chiou WF, Chou CJ, Shum AY, Chen CF. The vasorelaxant effect of evodiamine in rat isolated mesenteric arteries: mode of action. Eur J Pharmacol 1992;215:277–83.

[9] Sheu JR, Hung WC, Lee YM, Yen MH. Mechanism of inhibition of platelet aggregation by rutaecarpine, an alkaloid isolated from *Evodia rutaecarpa*. Eur J Pharmacol 1996;318:469–75.
[10] Moon TC, Murakami M, Kudo I, Son KH, Kim HP, Kang SS, et al. A new class of COX-2 inhibitor, rutaecarpine from *Evodia rutaecarpa*. Inf Res 1999;48: 621–5.
[11] Takada Y, Kobayashi Y, Aggarwal BB. Evodiamine abolishes constitutive and inducible NF-kappaB activation by inhibiting IkappaBalpha kinase activation, thereby suppressing NF-kappaB-regulated antiapoptotic and metastatic gene expression, up-regulating apoptosis, and inhibiting invasion. J Biol Chem 2005;280:17203–12.
[12] Choi YH, Shin EM, Kim YS, Cai XF, Lee JJ, Kim HP. Anti-inflammatory principles from the fruits of *Evodia rutaecarpa* and their cellular action mechanisms. Arch Pharm Res 2006;29:293–7.
[13] Jiang J, Hu C. Evodiamine: a novel anti-cancer alkaloid from *Evodia rutaecarpa*. Molecules 2009;14:1852–9.
[14] Wang T, Kusudo T, Takeuchi T, Yamashita Y, Kontani Y, Okamatsu Y, et al. Evodiamine inhibits insulin-stimulated mTOR-S6K activation and IRS1 serine phosphorylation in adipocytes and improves glucose tolerance in obese/diabetic mice. PLoS One 2013;8:e83264.
[15] Wang T, Wang Y, Kontani Y, Kobayashi Y, Sato Y, Mori N, et al. Evodiamine improves diet-induced obesity in a uncoupling protein-1-independent manner: involvement of antiadipogenic mechanism and extracellularly regulated kinase/mitogen-activated protein kinase signaling. Endocrinology 2008;149:358–66.
[16] Wang T, Wang Y, Yamashita H. Evodiamine inhibits adipogenesis via the EGFR-PKCalpha-ERK signaling pathway. FEBS Lett 2009;583:3655–9.
[17] Grygiel-Gorniak B. Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications—a review. Nutr J 2014;13:17.
[18] Bak EJ, Park HG, Kim JM, Kim JM, Yoo YJ, Cha JH. Inhibitory effect of evodiamine alone and in combination with rosiglitazone on in vitro adipocyte differentiation and in vivo obesity related to diabetes. Int J Obes (Lond) 2010;34:250–60.
[19] Yuan SM, Gao K, Wang DM, Quan XZ, Liu JN, Ma CM, et al. Evodiamine improves congnitive abilities in SAMP8 and APP(swe)/PS1(DeltaE9) transgenic mouse models of Alzheimer's disease. Acta Pharmacol Sin 2011;32:295–302.
[20] De Petrocellis L, Schiano Moriello A, Fontana G, Sacchetti A, Passarella D, Appendino G, et al. Effect of chirality and lipophilicity in the functional activity of evodiamine and its analogues at TRPV1 channels. Br J Pharmacol 2014;171:2608–20.
[21] Nguyen NV, Lee KR, Lee YJ, Choi S, Kang JS, Mar W, et al. Chiral high-performance liquid chromatographic separation of evodiamine enantiomers and rutaecarpine, isolated from *Evodiae fructus*. J Pharm Biomed Anal 2013;81–82:151–9.
[22] Davinelli S, Sapere N, Zella D, Bracale R, Intrieri M, Scapagnini G. Pleiotropic protective effects of phytochemicals in Alzheimer's disease. Oxid Med Cell Longev 2012;2012:386527.
[23] Yim CY, Mao P, Spinella MJ. Headway and hurdles in the clinical development of dietary phytochemicals for cancer therapy and prevention: lessons learned from vitamin A derivatives. AAPS J 2014;16:281–8.
[24] Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993;118: 401–15.
[25] Ueng YF, Don MJ, Peng HC, Wang SY, Wang JJ, Chen CF. Effects of Wu-chu-yu-tang and its component herbs on drug-metabolizing enzymes. Jpn J Pharmacol 2002;89:267–73.
[26] Sheu JR. Pharmacological effects of rutaecarpine, an alkaloid isolated from *Evodia rutaecarpa*. Cardiovasc Drug Rev 1999;17:237–45.
[27] Lee G, Elwood F, McNally J, Weiszmann J, Lindstrom M, Amaral K, et al. T0070907, a selective ligand for peroxisome proliferator-activated receptor gamma, functions as an antagonist of biochemical and cellular activities. J Biol Chem 2002;277:19649–57.
[28] Tachibana K, Kobayashi Y, Tanaka T, Tagami M, Sugiyama A, Katayama T, et al. Gene expression profiling of potential peroxisome proliferator-activated receptor (PPAR) target genes in human hepatoblastoma cell lines inducibly expressing different PPAR isoforms. Nucl Recept 2005;3:3.